ORGANIZATION OF MEMBRANE PROTEINS IN NORMAL AND ABNORMAL ERYTHROCYTES.

(EFFECT OF PHENYLHYDRAZINE)

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Dedicated to the

Everlasting

Memory

of

Nana Abba

(Prof. Abdul Aleem)

With love

to

My Sweet Parents

'A crutch I can lean upon anytime and everytime'

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CERTIFICATE

I certify that the work presented in this dissertation has been carried out by Miss AFSHAN NAHEED under my supervision and is suitable for the submission for the award of M.Phil. degree in Biochemistry.

Date: August 9, 1988

(M. Saleemuddin)
Professor of Biochemistry.

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CONTENTS

ABBREVIATIONS USED

Bis	...	N,N'-Methylene bisacrylamide
BSA	...	Bovine serum albumin
cm	...	Centimeter
Con AcBis	...	Concentrated acrylamide bisacrylamide
deg C	...	Degree Celsius
DTNB	...	Dithiobis (2-nitrobenzoic acid)
EDTA	...	Ethylene diamine tetracetate
hr(s).	...	Hour(s)
Kda	...	Kilodalton
M	...	Molar
mA	...	Milliampere
min(s).	...	Minute(s)
ml	...	Milliliter
mM	...	Millimolar
mm	...	Millimeter
N	...	Normal
nm	...	nanometer
PHZ	...	Phenylhydrazine
rbc(s)	...	Red blood cell(s)
SDS	...	Sodium dodecyl sulphate
SDS-PAGE	...	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
TEMED	...	N,N,N',N'-Tetramethylethylene diamine
Tris acetate	...	Tris (hydroxymethyl) aminomethane acetate
Tris HCl	...	Tris (hydroxymethyl) aminomethane HCl
ul	...	Microliter

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I. INTRODUCTION

THE HUMAN ERYTHROCYTE MEMBRANE

Human erythrocyte membrane is probably the best understood among the biological membranes in view of its easy availability, ease of preparation and characterization. It has long served as a convenient model for the testing of new concepts and methodologies in membrane biochemistry.

The erythrocyte membrane is composed of about equal amounts of lipids and proteins. The carbohydrate components of the red blood cell (rbc) membrane are confined to the exterior surface as oligosaccharide moieties of glycolipids and glycoproteins (Sweeley and Dawson, 1969). They are for the most part, responsible for the surface charge and surface determinant properties. It is well known that the rbcs have a large negative charge at their outer surface (Cook et al., 1961). Carboxyl groups of the sialic acid residues which are associated with the major rbc membrane glycoproteins,are responsible for most of the negative charge (Eylar et al.,1962).

The rbc membrane lipid composition is rather complex due to the occurance of several variants,both in polar and non-polar moieties of the molecule. The amount of total lipids in 10^{10} cells is about 5 mg which is comprised of about 30 % neutral lipids,60 % phospholipids and 10 % glycolipids (Cooper, 1969). Free cholesterol constitutes the major component of the neutral lipids in rbc. It is randomly distributed and present throughout the thickness of the rbc membrane (Higgins et al.,1973). Quantitatively, the most important fractions of human rbc lipids are the phospholipids or phosphatides. The phospholipids include glycero- and

sphingo-phospholipids. Lecithins, cephalins,phosphatidylserine and sphingomyelins are the four main subclasses of phospholipids. Human rbc membrane contains about 20 % sphingomyelin and 35 % phosphatidylcholine (White, 1973). Glycolipids constitute about 5 - 10 % by weight of the total lipids.

The first systematic separation of rbc membrane proteins was described by Fairbanks et al. (1971). These workers have designated the various polypeptide components by numbers, according to their mobility in polyacrylamide gels in presence of SDS. This nomenclature is now widely accepted.

Extensive literature is available on the nature and function of the principal polypeptides of the human erythrocyte membrane (Juliano, 1973; Haest,1982;Bennett,1985). Only a brief summary of the information on these polypeptides is presented below

Band 1 and 2 (Spectrin)- Spectrin is the major component of the human rbcs membrane extrinsic proteins and occurs as a stable dimer of two similar yet chemically distinct polypeptides. The two polypeptides, band 1 (240 Kda) and band 2 (220 Kda), are oriented as parallel and intertwisted chains (Marchesi,1979). Spectrin is believed to be atleast tetrameric and the association of dimers occurs end to end *in situ* (Morrow and Marchesi, 1981).

Band 2.1, 2.2 and 2.3 (Ankyrin)- Band 2.1 of 210 Kda connects band 2 of

membrane skeleton with band 3 of intrinsic domain (Morrow et al., 1980) and in situ it is found in the monomeric form(Bennett and Stenbuck,1980). Not much is known about the structure and associations of other ankyrin polypeptides corresponding to bands 2.2 (183 Kda) and 2.3 (105 Kda) (Siegel et al., 1980).

Band 3- Band 3 is the major intrinsic protein constituting about 50-60 % of the total intrinsic proteins. It is an inorganic anion transporting protein of 95 Kda (Cabantchik et al., 1975) and is believed to exist as dimers and to some extent as tetramers in the membrane (Nakashima et al., 1981). This protein traverses the rbc membrane and has an outer glycosylated domain and an intracellular domain, which is not glycosylated.

Band 4.1- The band 4.1, protein of 78 Kda, is involved in the interaction of spectrin with actin (Wolfe et al., 1980). The band 4.1 is a globular molecule of 6 nm diameter (Tyler et al., 1979). Each spectrin dimer has one binding site for band 4.1 located at the free end of dimer-dimer complex (Tyler et al., 1979).

Band 4.2- This is an intrinsic protein of unknown function consisting of a 4 x 72 Kda unit. The band 4.2 polypeptides remain associated with the cytoplasmic region of band 3. (Land and Nermut, 1980).

Band 4.9- Further putative component of membrane skeleton is the band 4.9 of 48 Kda. Oxidative treatment of cells results in cross-linking of band

4.9 with band 2 via disulfide bonds indicating the in situ proximity of the band (Liu and Palek, 1979).

Band 5- Band 5 polypeptide is also called actin. It is a component of the membrane skeleton and forms short oligomers containing about 10 molecules in filamentous form (Brenner and Korn, 1980). Each spectrin tetramer has two actin binding sites at the free end. This protein has been shown to play a major role in the shape change of rbcs.

Band 6- This is a monomer of glyceraldehyde-3-phosphate dehydrogenase. The enzyme is a tetramer of 35 Kda and binds to spectrin-actin complex as well as to band 3 (Yeltman and Harris, 1980; Murthy et al., 1981).

Band 7- Band 7 protein of 29 Kda has not yet been fully characterized and it is also not very clear as to where and how it is arranged in the erythrocyte membrane. The phosphatidyl serine transporter protein of human erythrocyte membrane has been shown to comigrate with the band 7 protein (Connor and Schroit, 1988).

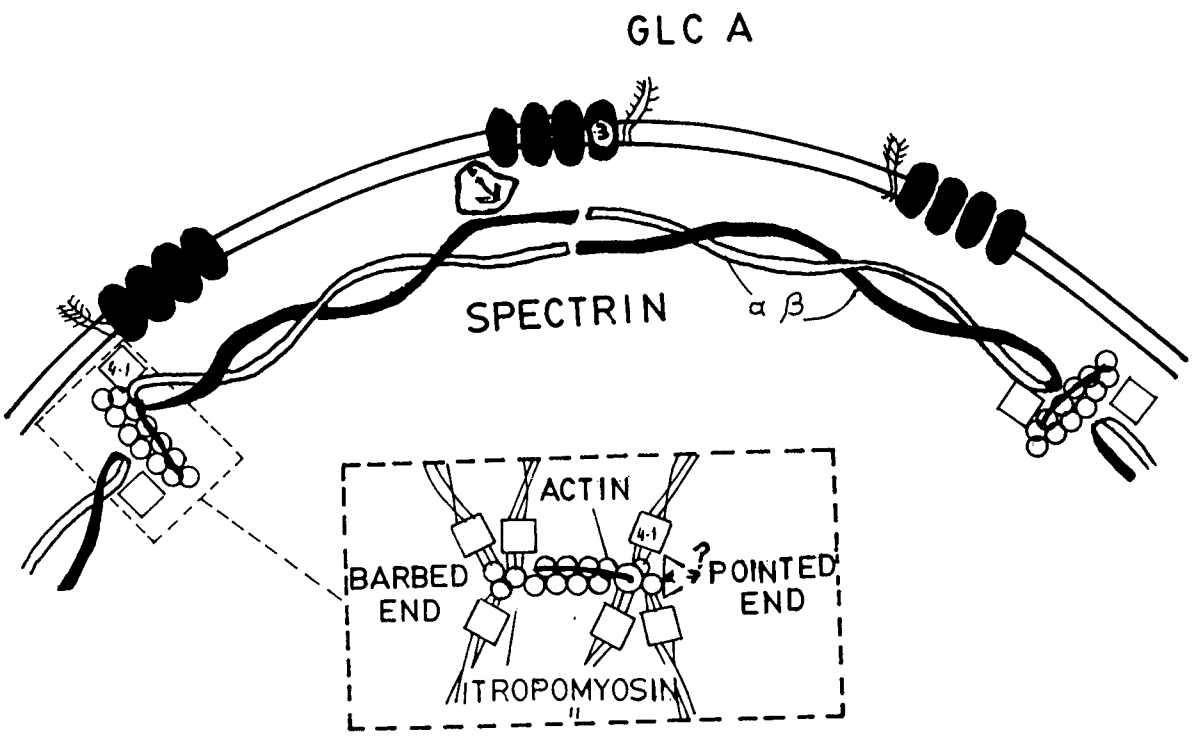
The human rbc membrane is decorated at the cytoplasmic surface with a densely packed spectrin-actin skeleton. From microscopic pictures and calculations it can be derived that the membrane skeleton covers 50-70 % of the membrane surface. The membrane skeleton was originally defined as the protein filamentous matrix of the same dimensions as the originating ghost that remains after extraction of the lipids and intrinsic proteins from intact erythrocytes or ghosts using Triton X-100 (Yu et al., 1973).

Subsequent,purification of the skeleton gave a lipid-poor (1-2 %) protein band containing besides spectrin (bands 1 and 2) a number of peptides and band 5 (Sheetz, 1979). This composition represents the minimal number of bands necessary to maintain stability of the skeleton. Stabilization of the skeleton components within the network probably occurs via non-covalent bonds of varying affinity as well as by electrostatic repulsions of negatively charged spectrin molecules.

Although the membrane skeleton highly restricts lateral mobility of intrinsic proteins, cytoskeleton is a highly shear deformeable lattice. Shear deformability of this lattice is evidenced by the extreme deformations of the cell in circulation as well as by deformations induced by drugs and other substances. The membrane skeleton can be viewed as being constructed around spectrin. Spectrin tetramers are involved in two indepedent classes of protein associations that are both essential for the final structure for (a) formation of the two dimensional meshwork underlying the lipid bilayer, by associations with actin oligomers, band 4.1 and possibly other spectrin molecules and (b) linkage of the spectrin/actin meshwork to integral membrane proteins via association with ankyrin and possibly band 4.1 (Fig.I).

Assembly of a meshwork requires some type of polymerization reaction leading to branched structures. A frequently discussed arrangement of proteins (Cohen et al., 1980; Brenner and Korn, 1980; Pinder and Gratzer 1983; Shen et al., 1984) involves a basic structural unit composed of actin filaments, containing 12-17 actin monomers and accessory proteins such as band 4.9 and tropomyosin,which are attached at the ends of multiple spectrin tetramer/band 4.1 complexes (Fig.I). These actin-spectrin

Fig. I. Schematic model of the human erythrocyte membrane skeleton. The barbed end of the actin filament is the fast-growing end while the pointed end is the slow-growing end.


GLC A
SPECTRIN
α β
4.1
ACTIN
4.1
?
BARBED END
POINTED END
TROPOMYOSIN

complexes can then assemble to form a branching polymeric structure by interconnections between the free ends of spectrin tetramers and other actin oligomers. It has also been proposed that self-assembly of spectrin into hexamers and higher order oligomers from erythrocyte membranes initiate polymerization of G-actin by a cytochalasin inhibitable reaction and thus these actin filaments have free fast-growing ends (Lin and Lin, 1979; Brenner and Korn, 1980; Pinder and Gratzer, 1983; Shen et al., 1984). In cells such as the developing erythrocytes and other tissues with related forms of spectrin, the spectrin-actin meshwork could have extended actin filaments cross-linked along their length by spectrin or other actin-binding proteins to form three dimensional structures. Presumably, during erythrocytes maturation,actin in excess of the final 5×10^5 copies per cell is lost, perhaps as the consequence of a band 4.1 severing activity (Pinder et al.,1984) and the red cell is left with a shell of short actin filaments adjacent to the membrane.

MEMBRANE PROTEINS IN ABNORMAL ERYTHROCYTES

Studies with hereditary hemolytic anemias in human and mice have introduced the beginings of genetics in the area of membrane skeletal proteins. It has been clearly demonstrated that abnormal or deficient membrane proteins lead to major defects in erythrocyte shape and mechanical stability. Hereditary spherocytosis and elliptocytosis are relatively common (approximately 1:5000) disorders of the erythrocyte membrane leading to fragile and/or abnormally shaped cells (Lux 1979; 1983; Palex and Lux, 1983). Erythrocytes accumulated in the spleen in both cases and anemia can be essentially cured by splenectomy. Hereditary spherocytosis

is in about 80 % of the cases transmitted as an autosomal dominant trait, while hereditary elliptocytosis is almost entirely autosomal dominant. These diseases were recognized initially on the basis of abnormally appearing erythrocytes, associated with varying degrees of anemia, that could not be explained by altered hemoglobin, glycolytic enzymes or autoimmune features. A distinctive feature of the spherocytosis group is that these cells are more susceptible to hypotonic lysis, presumably, due to a decreased surface area/volume ratio.

Pyropoikilocytosis is a rare recessively inherited variant of hereditary elliptocytosis and is characterized by erythrocytes that fragment and attain bizarre shapes after warming to 45-46 deg C which is about 4 deg less than the disintegration temperature for normal erythrocytes (Chang et al., 1979). Spectrin isolated from these cells denatures at the same temperature of 45-46 deg C based on change in the ORD spectrum, while normal spectrin denatures at 49-51 deg C. Spectrin from patients with pyropoikilocytosis also has a lowered affinity for the dimer-tetramer equilibrium (Liu et al., 1981; Knowles et al., 1983). It is likely that a change in spectrin amino acid sequence is responsible for these features, since an altered tryptic cleavage has been detected in an 80,000 Mr domain of the alpha chain (the portion of the molecule involved in dimer self-association,with the adjacent beta chain).A similar defect has also been observed in some patients with mild hereditary elliptocytosis (Liu et al., 1982; Coetzer and Zail, 1982; Evans et al., 1983).

High affinity ankyrin binding sites are reduced by 50% in erythrocyte

membranes in two families with an elliptocytosis type anemia (Agre et al., 1981). The cytoplasmic domain of band 3 purified from the defective membranes binds to ankyrin normally. These erythrocytes may have an altered arrangement of band 3 molecules rather than a defect in the binding site itself.

It is also possible that spectrin binding defects play a role in hemolysis in other forms of anemia. The binding of ankyrin to spectrin is sensitive to alkylation of a sulfhydryl group on ankyrin (Bennett and Stenbuck,1980).It is possible that metabolic disorders such as glucose-6-phosphate dehydrogenase deficiency, which leads to lowered levels of reduced glutathione, could result in the oxidation of ankyrin sulfhydryl groups. The ability of ankyrin to bind to spectrin would be compromised and possibly result in increased erythrocyte fragility.

Another mechanism for the disease is the absence or decreased quantity of these proteins due to abnormal synthesis or instability of the product.The first example of deficient proteins came from the strains of mutant mice that were developed at the Jackson Laboratory (Russell, 1979; Bernstein, 1980). SDS-gels of ghosts membranes from these strains revealed a striking decrease in the quantity of spectrin, and the extent of deficiency correlated with severity of the anemia (Greenquist et al., 1978; Lux et al., 1979).

Decreased spectrin results from different mechanisms,depending on the strain of mouse (Bodine et al.,1984). One mutant (nb/nb) produced no stable ankyrin, but did synthesize normal amounts of spectrin. Thus spectrin deficiency in nb/nb mice is secondary to lack of ankyrin.These mice still

have some membrane associated spectrin,indicating some spectrin/actin meshwork assembly and/or membrane associations of spectrin can occur in the absence of ankyrin.Another strain (ja/ja) lacks the ability to synthesize beta subunit of spectrin, although spectrin alpha chain mRNA was present in elevated amounts.Lack of the beta subunit,which has the ankyrin binding site, thus prevents assembly of the spectrin/actin meshwork since actin-binding requires the presence of both subunits (Calvent et al., 1980; Cohen and Langley, 1984).

Examples of markedly deficient protein 4.1 and spectrin have also been reported in some human hemolytic anemias. A form of elliptocytosis with abnormal erythrocyte morphology and increased fragility has been associated with lack of band 4.1 (Mueller and Morrison, 1981; Tchernia et al., 1981). The parents had 50 % of the normal complement of band 4.1 and were asymptomatic with mildly abnormal erythrocytes. The affected individuals had a complete absence of band 4.1. It is not clear whether band 4.1 is lacking due to ineffective synthesis,abnormal degradation,decreased association with membranes,or if band 4.1 is present but is of different molecular weight.

The amount of reduction in spectrin in patients with spherocytosis correlated closely with severity of the disease and with the reduction in membrane stability measured by the osmotic fragility assay, while other membrane proteins were essentially unchanged.Patients with a 10-20% spectrin deficiency have a mild, compensated anemia that may be subclinical, while patients with 20-40 % reduction in spectrin are moderately to severely anemic,usually require splenectomy and are detected in children. The

molecular basis for spectrin deficiencies is likely to be diverse and by analogy with the mutant mice fall into two major categories, (a) Unstable spectrin due to a defect in assembly or intrinsic susceptibility to protease and (b) Lowered synthesis of spectrin due to problems with mRNA synthesis or stability.

UNSTABLE HEMOGLOBINS

Structural abnormalities of the hemoglobin molecule may result in instability through a number of different mechanisms and are a rare cause of congenital hemolytic anemia. In many instances instability leads to denaturation and precipitation of the abnormal hemoglobin within the cell,producing amorphous inclusions known as Heinz bodies (Table 1). These attach themselves to the inner surface of the rbc membrane making the cells less flexible and impede their passage through microcirculation. Heinz bodies are much more numerous after splenectomy and it is likely that cells are normally hindered in the spleen where Heinz bodies can be snipped out by the splenic macrophages or the cells can be removed altogether.

In most instances the hemoglobin instability results from a single or small number of amino acid substitutions in a critical area of the molecule, for example, at a heme binding site or at a point of contact between individual globin chains (Table 2).

The clinical features of an unstable hemoglobin disorder are of a hemolytic anemia with splenomegaly. Exacerabation may occur during infec-

Table 1. Mechanism and Effect of Heinz Body Formation

(White and Dacie, 1971)

Postulated pathway	*In vitro* observation
Access of water to heme, generation of HO2 radical and H_2O_2.	Generation of methemoglobin.
Oxidation of F9 cysteines and GSH.	Low GSH, blockade of Hb, sulfhydryl groups.
GSH binds onto F9 cysteins.	Stimulation of HMP shunt.
Abnormal chains loose heme.	Free heme catabolized to dipyrroles.
Dissociations of tetramer into beta and alpha monomers, precipitation of abnormal chain (Heinz Body).	Free soluble alpha chain monomers in lysates.
Heinz body binds to membrane sulfhydryl groups.	Reduction in titrable membrane sulfhydryl groups.
Rigid membrane and abnormal membrane function.	Increased cation permeability.
Splenic sequestration and phagocytosis of Heinz body containing cell.	Electron micrographic evidence.
Removal of Heinz bodies by spleen from cells sustaining minor damage without destruction of rbcs. Removal by sequestration and phagocytosis in reticuloendothelial system generally of red cells sustaining major damage.	rbcs have low hemoglobin concentration, also the unstable hemoglobin are synthesized in amounts greater than can be detected in the peripheral blood; Heinz bodies seen only after splenectomy.

GSH --- Reduced Glutathione
HMP --- Hexose monophosphate shunt pathway

Table 2. Some Naturally Occuring Unstable Hemoglobins

Name	Substitution	Helical site	Abnormality	Reference
Beta Chain Variants				
Boras	Leu ----- Arg	F4	Heme contact	Steadman et al. (1970) Hollender et al. (1969)
Bristol	Val ----- Asp	E11	Heme contact	Steadman et al. (1970)
Freiburg	Val deleted	B5	Deletion	Jones et al. (1966)
Genova	Leu ----- Pro	B10	Pro breaks alpha helix	Sansone et al.(1967)
Gun Hill	Deletion of 5 amino acids on F and FG helix.	-	Deletion	Bradley et al.(1967)
Hammersmith	Phe ----- Ser	CD1	Heme contact	White & Dacie (1971) Dacie et al. (1967) Huehns (1970) Grimes et al. (1964)
Koln	Val ----- Met	FG5	Heme contact	Carrell et al.(1966)
Leiden	Glu acid deleted	A3 or A4	Deletion	De Jong et al.(1968)
Olmsted	Leu ----- Arg	H19	Non polar --- polar	Lorkin and Lehmann (1970)
Philly	Tyr ----- Phe	C1	Weakens alpha 1 beta 1 contact	Rieder et al.(1969)
Riverdale Bronx	Gly ----- Arg	B6	Non polar --- polar	Rannay et al. (1968) Zalusky et al.(1970)
Sabine	Leu ----- Pro	F7	Heme contact Pro breaks F helix.	Opfell et al. (1968)

.... contd.

Table II (contd.)

Name	Substitution	Helical site	Abnormality	Reference
Santa Ana	Leu ----- Pro	F4	Heme contact Pro breaks F helix	Opfell et al. (1968)
Seattle	Ala ----- Glu	E20	Glu interacts with His EF1.	Huehns et al. (1970)
Shepherds Bush	Gly ----- Asp	E18	Non-polar-polar	White et al. (1970)
Sogn	Leu ----- Arg	A11	Non polar --- polar	Monn et al. (1968)
Sydney	Val ----- Ala	E11	Heme contact	Carrell et al.(1967)
Tacoma	Arg ----- Ser	B12	weakens alphal betal contact	Perutz and Lehmann, (1968)
Toulouse	Lys ----- Glu	E10	Heme Contact	Rosa et al. (1969)
Wien	Tyr ----- Asp	H8	Non polar --- polar	Perutz and Lehmann (1968)
Zurich	His ----- Arg	E7	Heme Contact	Huehns (1970)
Alpha chain variants				
Ann Arbor	Leu ----- Arg	F1	Non polar --- polar	Rucknagel et al. (1968)
Bibba	Leu ----- Pro	H19	Pro breaks H helix.	Kleihauer et al. (1968).
Dakar	His ----- Gly	G19	Stabilizes G and B helices	Rosa et al. (1968)
Etobicoke	Ser ----- Arg	F5	Non polar --- polar	Crookston et al. (1969)
L-Ferrara	Asp ----- Gly	CD5	Unknown	Huehns (1970)
Hasharon	Asp ----- His	CD5	Unknown	Charache et al. (1967)
Torino	Phe ----- Val	CD1	Heme contact	Beretta et al.(1968)

tion in association with oxidant drugs such as sulfonamides, nitrofurantoin or nalidixic acid, as the unstable hemoglobin is particularly sensitive to oxidant stress and hemoglobinuria may occur. The diagnosis of an unstable hemoglobin may be difficult, especially if the defect is mild. The most characteristic finding is an abnormal heat stability pattern: heating a dilute solution of hemoglobin to 50 deg C for 15 min. causes most of the unstable hemoglobin to precipitate.Hemoglobin electrophoresis may be useful but will be normal if the substitutions involve no change, in the overall charge of the molecule (Bunch, 1985).

EFFECT OF PHENYLHYDRAZINE

A number of hemolytic agents induce non-indegenous redox processes in erythrocytes. These drugs lower the circulating rbc population in susceptible individuals by reducing their life span and also cause membrane deformabilty (Clark et al.,1983). Persons with genetic deficiencies in key enzymes viz.glucose-6-phosphate dehydrogenase,glutathione reductase, etc. that are involved in red cell metabolism are particularly susceptible to the induction of hemolytic anemia by these redox drugs. Of these agents, phenylhydrazine (PHZ) is well known and has been subjected to a large number of investigations.

PHZ is a potent redox active drug that can induce hemolytic anemia even in individuals without any erythrocytic enzyme deficiency (Beutler, 1969). Observations in the last century indicated that PHZ could induce dramatic changes in erythrocytes both in vitro as well as in vivo.

Hoppe-Seyler (1885) reported that blood from rabbits treated with PHZ was brown in colour and that addition of PHZ to the suspended erythrocytes gave them also a brown colouration. Subsequently, Heinz (1890) found that mixing either nucleated (from cold-blooded animals) or non-nucleated erythrocytes with PHZ turned them green-brown. Heinz (1890) also discovered that inclusion bodies were formed in erythrocytes exposed to PHZ.

The hemolytic effects of PHZ, a reducing agent,may thus be viewed as a consequence of its autoxidation and the subsequent oxidation of the essential sulfhydryl groups of enzymes and membrane proteins. The cellular and chemical responses of the rbcs to this agent have been thoroughly investigated. Oxyhemoglobin forms methemoglobin by PHZ induced processes and the reduction of methemoglobin to deoxyhemoglobin (in anaerobic systems) or the formation of HbO2 (in aerobic environments) can also be induced to occur. The oxidation of PHZ leads to the formation of a number of products including benzene (Beaven and White, 1954), hydrogen peroxide (Cohen and Hochstein, 1964), superoxide anion (Goldberg and Stern, 1975) and phenyl radicals (Hill and Thornalley, 1981). Some other species suspected of being involved in the reaction of hemoglobin with PHZ include phenyldiazene (Beaven and White, 1954) and phenylhydrazyl (Mishra and Fridovich,1976). It has also been reported that oxyhemoglobin and myoglobin react with PHZ to yield a derivative of hemoglobin containing N-phenylprotoporphyrin in which the heme group is modified. Moreover, the identification of phenyliron (III) porphyrins in PHZ modified metmyoglobin has supported the elucidation of the mechanism of hemoglobin modification.

PHZ is readily permeable across the rbc membrane and has been shown to cause a number of effects. Thus its administration in vivo may lead to the production and accumulation of hydrogen peroxide in amounts above the detoxifying capacity of cellular protective mechanism (Cohen and Hochstein 1961; 1965). Additionally, PHZ has been shown to generate superoxide (Valenzuela et al., 1977; Mishra and Fridovich, 1976; Jain and Hochstein, 1979), hydroxyl radicals (Mishra and Fridovich, 1976) and phenylradicals (Goldberg and Stern, 1977). The cytotoxicity of PHZ need not necessarily involve phenylradicals since hydrazine itself has effects on erythrocytes similar to those produced by PHZ (Jain and Hochstein,1979). Itano et al. (1975, 1976) have further suggested that the formation of ferrichrome, a product of phenyldiazene and ferrihemoglobin interaction may be involved in PHZ induced hemolysis.

Jain and Hochstein (1980) have suggested that none of the factors associated with intracellular oxidative alterations e.g. glutathione, methemoglobin, Heinz bodies and hemichrome are altered in cells exposed to PHZ in vivo.The lack of such changes suggests that intracellular peroxide detoxifying mechanisms are functioning adequately in these cells despite their shorter life spans.

Spectrin is a major protein constituent of erythrocyte membranes and is presumably involved as a determinant of cell shape and deformability (Singer,1974;Greenquist and Shohet,1975). A decrease in the membrane content of spectrin and an increase in high molecular weight proteins and in the polymerized products of lipid peroxidation, might well be expected to

alter the rigidity and the life span of the affected cells (Heusinkveid et al.,1977; Dobretsov et al., 1977). PHZ localized at membrane sites undergoes autoxidation to form species capable of initiating lipid peroxidation. The membrane changes which follow might form the basis for enhanced sequestration and decreased survival of even reticulocytes which exhibit no intracellular oxidative damage (Jain and Hochstein, 1980).

II. MATERIALS AND METHODS

Most of the chemicals used in the present studies were obtained from various sources as shown below. Other chemicals and reagents used were of analytical grade or the best grade available commercially. Glass distilled water was used in all the experiments.

CHEMICALS	SOURCES
Acrylamide	Sigma Chemical Co., U.S.A.
Ammonium persulphate	May & Baker Ltd., England
Bovine serum albumin	Sigma Chemical Co., U.S.A.
Coomassie brilliant blue R-250	Sigma Chemical Co., U.S.A.
Dithiobis (2-nitro benzoic acid)	Sigma Chemical Co., U.S.A.
Ethylene diamine tetraacetate	B.D.H., India
Glutathione	Biogen, India
Iodoacetate	Sigma Chemical Co., U.S.A.
Beta-Mercaptoethanol	Sigma Chemical Co., U.S.A.
N,N'-Methylene bisacrylamide	B.D.H., India
1,10,Ortho-phenanthroline	E. Merck, India
Phenylhydrazine	Riedel-de Haen Ag, W.Germany
Pyronin Y	Sigma Chemical Co., U.S.A.
Sodium dodecyl sulfate	Sigma Chemical Co., U.S.A.
N,N,N',N'-Tetramethylethylene diamine	B.D.H., India
Tris (hydroxymethyl) amino methane.HCl	Sigma Chemical Co., U.S.A.

Preparation of Erythrocyte Ghosts: Outdated human blood obtained from the local blood bank was used. Ghosts were prepared by the method of Fairbanks et al. (1971) which in turn is based on the principle of hypotonic lysis described by Dodge et al. (1963).25 ml of blood was diluted with an equal volume of cold 0.9% NaCl. The suspension was spun at 100 g for 10 min. at 37 deg C and the supernatant and 'buffy coats' were aspirated. The packed cells were washed three times in 0.9% NaCl. 1 ml of the washed cells were hemolysed by mixing them rapidly with 40 ml of cold 5 mM Tris-HCl buffer pH 7.4. The suspension was centrifuged at 12,000 g for 30 min. at 4 deg C. The resultant deep red supernatant was aspirated leaving the red translucent pellet of packed ghosts over minute opaque cream coloured "buttons". Removal of the latter at this stage was essential for minimizing contamination of ghosts with proteases. Each centrifuge tube was tilted and rotated to allow the loose ghost pellet to slide off the tightly packed button which could be then aspirated with little loss of ghosts. The preparation obtained after few additional washes was used. For electrophoresis the ghosts were dissolved in a solution containing 1 % SDS, 5-10 % sucrose,10 mM Tris HCl (pH 8),1 mM EDTA (pH 8),and 10 µl/ ml of Pyronin Y (tracking dye).

Polyacrylamide Gel Electrophoresis: The overall approach was that of Fairbanks et al.(1971) with slight modification. For gel polymerization the alternative procedure of Davis et al. (1964) was followed. Compositions for stock solutions, buffers and gels are as follows.

A. Stock solutions

Con AcBis
Acrylamide (40 g)
Bis (1.5 g)
H_2O to 100 ml

20 % (w/w) SDS

1.5 % (w/v) ammonium persulfate

0.5 % (v/v) TEMED

10 x Buffer (pH 7.4)
1.0 M Tris 40 ml
2.0 M Sodium acetate (10 ml)
0.2 M EDTA (10 ml)
Acetic acid (pH 7.4)
H_2O to 100 ml

B. Electrophoresis buffer (per liter)
10 x Buffer (100 ml)
20 % SDS (10 ml)
H_2O (890 ml)

C. Gels (per 40 ml of solution 5.6 % in acrylamide)
Con. AcBis (5.6 ml)
10 x buffer (4.0 ml)
20 % SDS (4.0 ml)
H_2O (20.4 ml)
1.5 % ammonium persulfate (4.0 ml) prepared freshly at the time of use.
0.5 % TEMED (2.0 ml)

Concentrated stock solution was added in the proportions given in C and this was added to 9 cm long glass tubes of 6 mm diameter that were previously cleaned by washing with hot SDS. Each column (height 85 mm) was overlaid gently with 20 ul distilled water. When polymerization was complete the top of the gels were overlaid with about 0.5 ml of electrophoresis buffer. The gels were left to stand for atleast 12 hrs. Electrophoresis was performed with a current of 4 mA/tube. The tubes were removed from the electrophoresis apparatus as the tracking dye bands reached 75 mm mark from their origin.

The gels were stained for protein with coomassie brilliant blue R-250 (Fairbanks et al., 1971). They were placed in glass test tubes (height 15 cm and diameter 1.5 cm) to which were added, the fixing,staining and destaining solutions. The solutions were kept overnight at 37 deg C or at room temperature in a solution of 0.025 % coomassie brilliant blue R-250 in 25 % isopropyl alcohol and 10 % glacial acetic acid. Gels were finally destained in 10 % glacial acetic acid.

Membrane Protein Estimation: The membrane protein were estimated as described by Haest et al. (1978) using the procedure of Lowry et al. (1951). Red cell membrane was dissolved in 0.2 % SDS (1:1) and suitable aliquots of this solution were diluted to 1 ml with distilled water. To this was added 5.0 ml of freshly prepared copper reagent (prepared by mixing in 1:50 ratio of 0.5 % (w/v) copper sulphate in 1 % sodium potassium tartarate and 2.0 % (w/v) sodium carbonate in 0.1M sodium hydroxide). After incubation at room temperature for 10 min, 0.5 ml of 1N Folin-phenol reagent was added and the tubes were instantly Vortexed. Absorbance of the developed blue color was measured after 30 min at 660 nm against a reagent blank in Spectronic 20 spectrophotometer. BSA was used as a reference standard.

Determination of Membrane Sulfhydryl Groups:The overall approach was that of Haest et al.(1978). 0.1 ml of freshly prepared ghosts or those treated with PHZ were mixed with 0.5 ml of 20 % SDS, and 0.5 ml of 1 mM solution of 5,5'-dithiobis (2-nitrobenzoic acid) was added. The tubes were incuba-

ted at 37 deg C for 1 hr and the absorbance recorded at 412 nm. For the determination of sulfhydryl content of protein, glutathione was used as a reference standard.

Cross-linking of rbc Membrane Protein: The membrane protein cross-linking was performed using copper-orthophenanthroline as descrined by Steck (1972) and modified by Liu and Palek (1979).

A stock solution was prepared by dissolving 20 mg of 1, 10-ortho-phenanthroline and 5 mg of copper sulphate in 2 ml distilled water. This solution was diluted hundred fold to get the working reagent which contained 50 mM 1,10,ortho-phenanthroline and 10 mM copper sulphate.

0.5 ml of rbc membrane in 0.5 ml of 5 mM Tris-acetate buffer, pH 8.0, was mixed with 1 ml working reagent and incubated at 0 deg C for 20 min. The supension was diluted by adding 40 ml of 5 mM Tris-acetate buffer, pH 8.0, to stop the reaction. The oxidized membranes were collected by centrifugation for 30 min at 15,000 g.

Proteolysis of Human rbc Membrane: Human rbc membrane was prepared by the method of Dodge et al. (1963) and cross-linked by copper-orthophenanthroline mixture (Steck, 1972). One volume of the membrane was incubated at 37 deg C with five volumes of 5mM Tris-acetate buffer, pH 8.0, for 1 hr, 2 nrs or 3 hrs. The reaction was stopped by adding 40 ml of Tris-acetate buffer, pH 8.0. The ghosts were isolated by centrifugation at 4 deg C for 20 min. The membranes were dissolved in tracking dye (without beta-mercaptoethanol) in 1:1 ratio and kept for 5 min in boiling water bath.

Characterization of the Polymeric Material: The separation gels were cast in the glass tubes upto height of 7 cm as described in text. The upper gels were prepared by mixing 100 mg agarose, 0.5 ml of 20 % SDS and 1 ml of beta-mercaptoethanol in 10 ml of distilled water and heated to 40 deg C. The mixture was poured on the tops of the already polymerized gels (upto 1 cm).

The electrophoresed unstained gels containing the large molecular weight protein adducts were sliced from discs and placed on the top of the new gels and subjected to re-electrophoresis.

III. RESULTS

The oxidative stress caused by PHZ in erythrocytes and other cells is well documented. In our attempt to understand the nature of oxidative damage caused to the erythrocyte membrane by PHZ,the disappearance of the sulfhydryl groups were studied.As shown in Fig. II, treatment with PHZ at 1 mM and 2 mM concentrations resulted in a very significant decrease in the membrane sulfhydryl groups. Thirty min. treatment with 1 mM PHZ resulted in the loss of about 60 % membrane sulfhydryl groups as measured by DTNB procedure described by Haest et al. (1978). Increase in the incubation period to 60 min., or use of 2 mM PHZ resulted in only a small further increase in the loss of membrane sulfhydryl groups.

As shown in Fig. III, treatment of human rbc with concentrations of PHZ upto 10 mM resulted in small further decrease in membrane sulfhydryl groups. Sixty min., incubation, at all the concentrations tested produced slight but consistent enhancement in the further loss of membrane sulfhydryl groups. At very high concentrations of PHZ, however, there was very large precipitation of hemoglobin and the preparation of the membranes containing small amounts of hemoglobin was difficult.

As shown in Fig.IV, treatment with 10 mM iodoacetate which is known to cause not more than 50% reduction in the rbc sulfhydryl groups, led to a small further reduction of membrane sulfhydryl groups (Haest et al., 1981). It should be mentioned that complete removal of hemoglobin from the erythrocyte membranes was very difficult after PHZ treatment.

Effect of Phenylhydrazine on Membrane Proteins: The polyacrylamide gel electrophoresis performed in presence of SDS of the human rbc membrane

Fig. II. Effect of Phenylhydrazine on Membrane Sulfhydryl Groups:

Human erythrocytes were washed three times with normal saline. These washed rbcs were treated with 1 mM or 2 mM of phenylhydrazine for 30 or 60 min., at 37 deg C as described in the text. After incubation, the erythrocytes were hemolyzed and membranes prepared as described earlier in the text. Membrane sulfhydryl groups were determined according to the method of Haest et al. (1981). The membrane protein was estimated by the method of Lowry et al. (1951). Symbols denote (●) 30 min., and (▲) 60 min., incubation at 37 deg C.

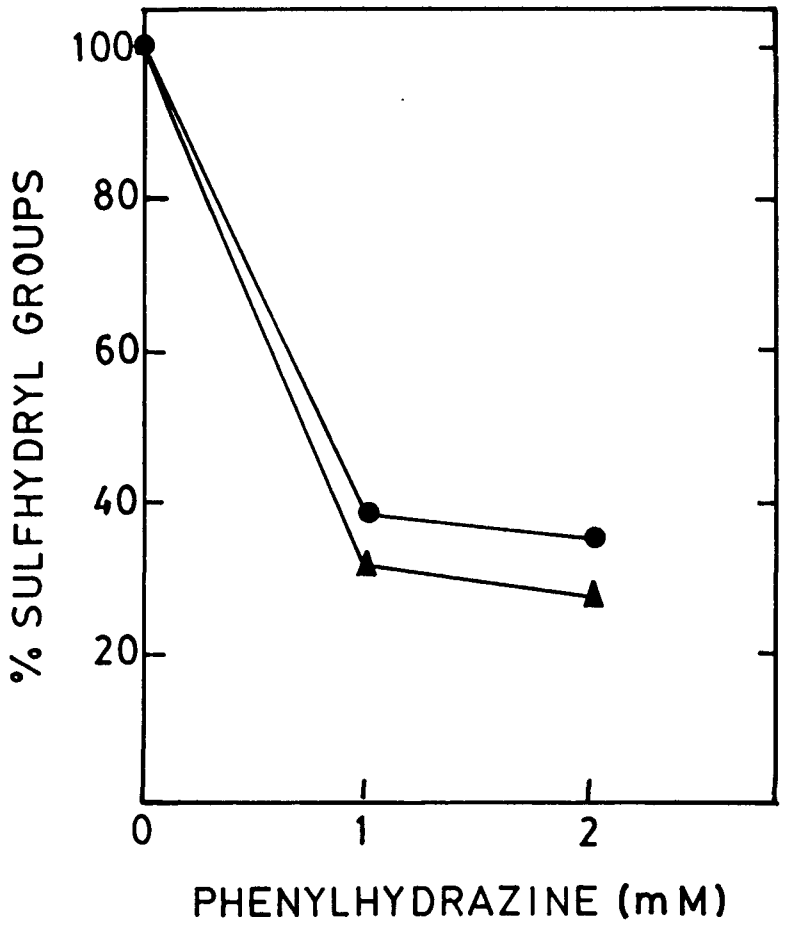

100
80
60
40
20
% SULFHYDRYL GROUPS
0
1
2
PHENYLHYDRAZINE (mM)

Fig. III. Effect of Phenylhydrazine Concentration on the Membrane Sulfhydryl Groups:

Washed human rbcs were treated with 1-10mM phenylhydrazine for 30 min.at 37 deg C and the membranes were prepared using the method of Dodge et al. (1963) as described in text. The membranes were separately assayed for sulfhydryl groups. Symbols denote (●) 30 min.and (O) 60 min. incubation at 37 deg C.

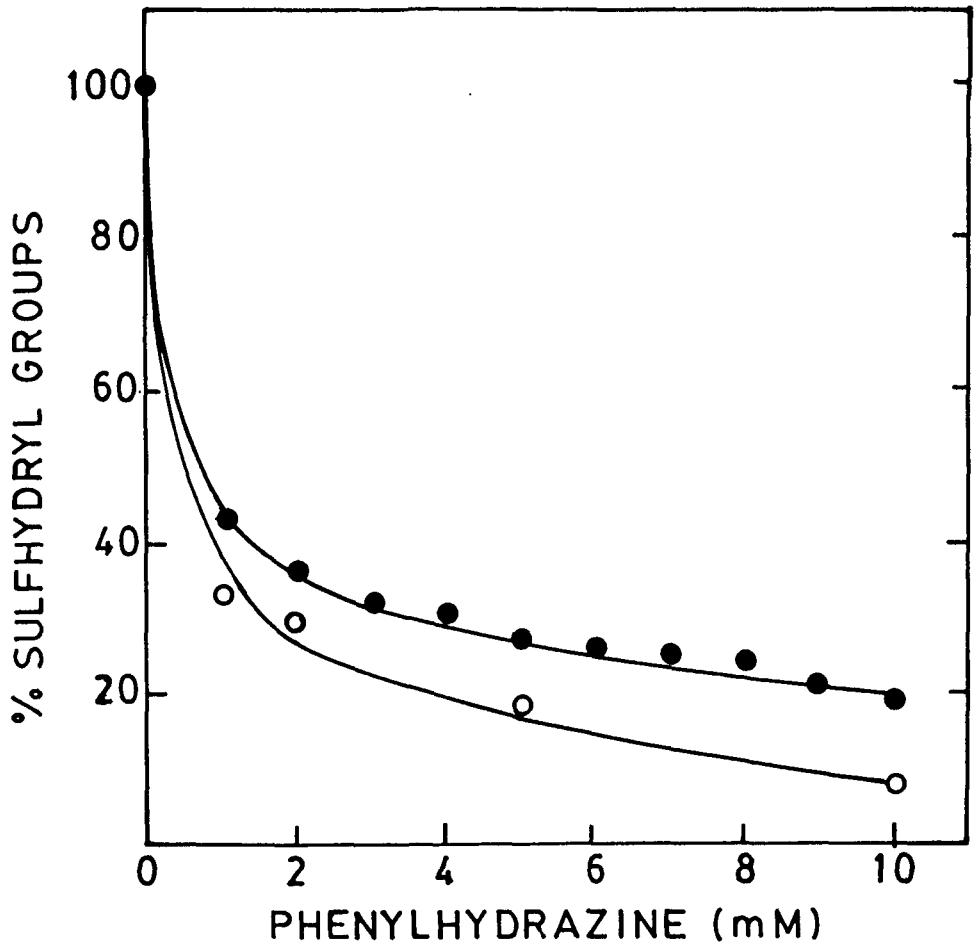
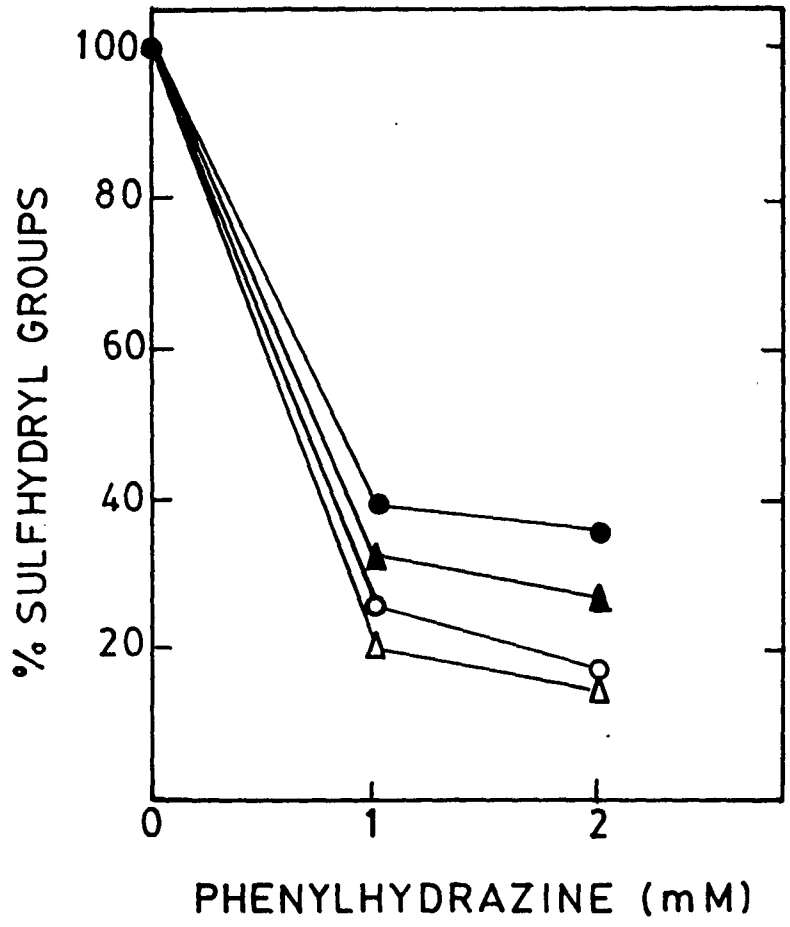

100
80
60
40
20
% SULFHYDRYL GROUPS
0
2
4
6
8
10
PHENYLHYDRAZINE (mM)

Fig. IV. Effect of Phenylhydrazine and Iodoacetate on rbc Membrane

The membrane of phenylhydrazine-treated rbcs were isolated and incubated with or without 10 mM iodoacetate at pH 8.0 for 30 min. at 37 deg C, essentially following the method of Fischer et al. (1978). The reaction mixture was centrifuged at 4 deg C and the pellet containing membrane was isolated and the sulfhydryl groups and protein content determined according to the method of Haest et al. (1981) and Lowry et al.(1951) respectively. Symbols denote (●) Phenylhydrazine-treated for 30 min. (▲) Phenylhydrazine-treated for 60 min. (o) Phenylhydrazine-treated 30 min. + 10 mM iodoacetate (Δ) Phenylhydrazine-treated 60 min. + 10 mM iodoacetate.


100
80
60
40
20
0
1
2
% SULFHYDRYL GROUPS
PHENYLHYDRAZINE (mM)

yielded pattern similar to that reported by Fairbanks et al.(1971), (Fig. V). Treatment with PHZ, however,resulted in significant formation of aggregated material that failed to enter the gel (Fig. VI). In addition, significant amount of hemoglobin could be seen in some of the gels. A very significant increase in the coomassie blue stainable material in the region of band 2.1 and some increase at the 4.1 region was also observed. Treatment with 2 mM of PHZ also yielded similar pattern except that the bands became more diffuse and there was further increase in the staining at 2.1 and 4.1 regions. When the electrophoresis was performed in the presence of beta-mercaptoethanol, however, the aggregated material on top of the gel almost disappeared, indicating the involvement of disulfide bridges in the formation of the aggregate (Fig. VII).

Characterization of the aggregate: Since the polymeric material formed as a result of PHZ treatment could be cleaved by using a sulfhydryl reagent, an attempt was made to characterize the polypeptides that constituted the aggregated material. For this purpose, the top regions of the unstained gels containing membranes of the PHZ-treated cells were cut and fragmented.The small fragments were added on to a second gel and the SDS PAGE electrophoresis was performed in presence of beta-mercaptoethanol. As shown in Fig.VIII, bands corresponding to spectrin polypeptides and those that migrated between 4.2 and 5 region were detected. Only traces of coomassie blue stainable material, if any, was detected at the region corresponding to band 3 and other major polypeptides.

Fig. V. The Polypeptide Composition of Human rbc Membrane

The human rbcs, were hemolysed by hypotonic treatment and washed to completely remove hemoglobin. The ghosts were solubilized in SDS and electrophoresis was performed essentially according to the procedure of Fairbanks et al. (1971), except that beta-mercaptoethanol was not included. The protein bands was numbered according to the nomenclature given by Fairbanks et al. (1971).

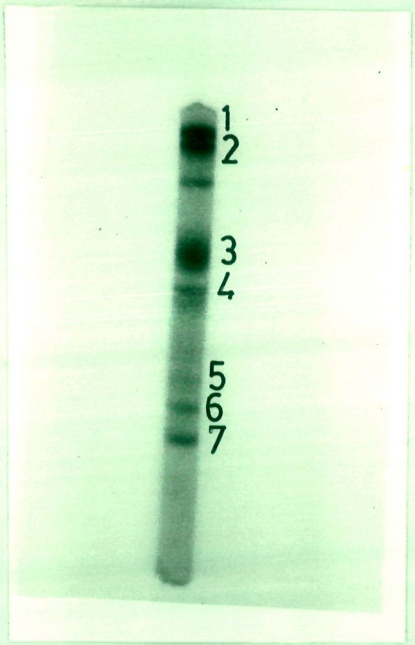

1
2
3
4
5
6
7

Fig. VI. Polypeptide Composition of Phenylhydrazine-Treated Erythrocyte Membranes

The washed human rbcs were treated with 1 mM (A) or 2 mM (B) of phenylhydrazine for 30 and 60 min., as described earlier and subjected to polyacrylamide gel electrophoresis in absence of beta-mercaptoethanol. Gels a control; Gels b phenylhydrazine-treated, 30 min.; Gels c phenylhydrazine -treated 60 min.

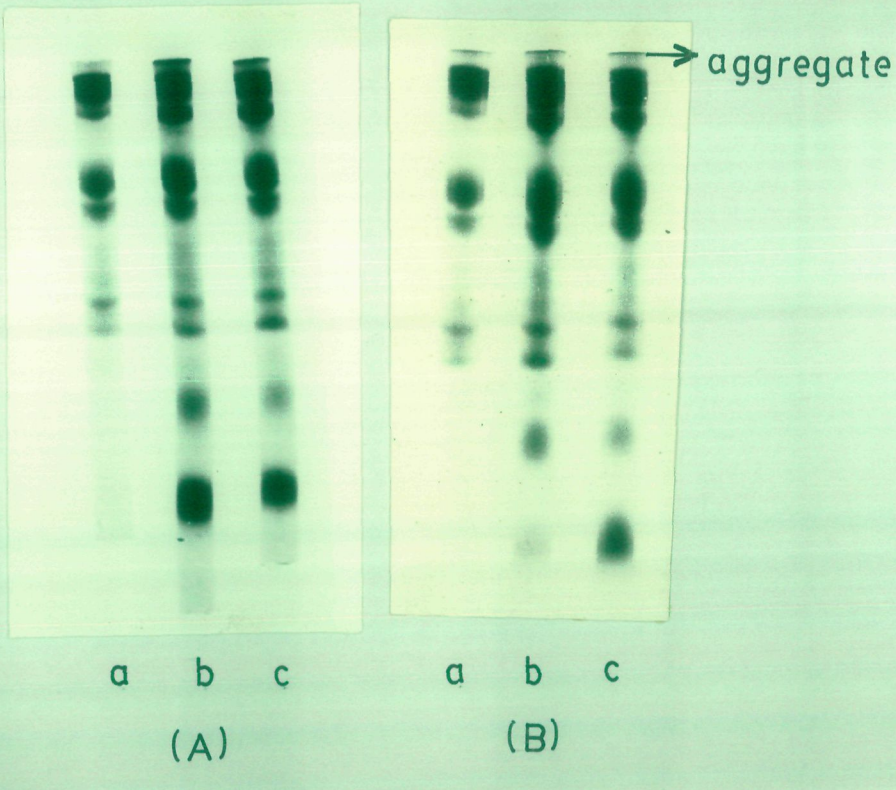

aggregate
a b c
a b c
(A)
(B)

Fig. VII. Polypeptide Composition of Phenylhydrazine-Treated Erythrocyte Membranes Subjected to SDS-PAGE in Presence of Beta-mercaptoethanol.

Washed human rbcs were treated with 2 mM of phenylhydrazine for 60 min. as described earlier. Polyacralamide gel electrophoresis was carried out of the membranes prepared in presence of 40 mM beta-mercaptoethanol (Fairbanks et al.,1971). The electrophoresis buffer also contained 40 mM beta-mercaptoethanol. Gel A control; Gel B 2 mM phenylhydrazine-treated, 60 min.; Gel C 2 mM phenylhydrazine-treated, 60 min., (electrophoresis was made in absence of beta-mercaptoethanol).

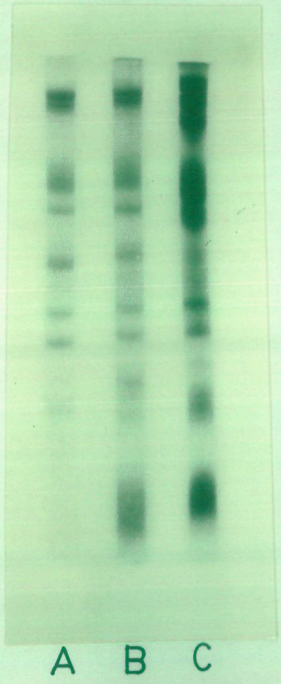
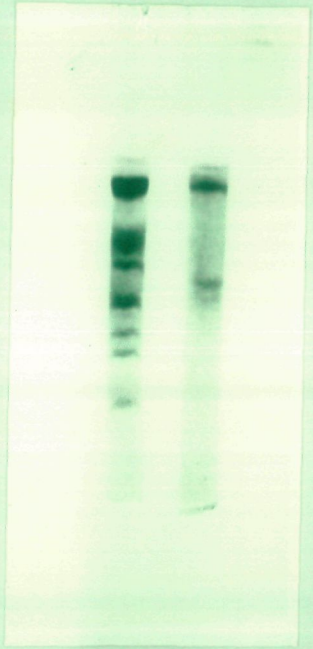

A
B
C

Fig.VIII. Determination of the Composition of the High Molecular Weight Cross-linked Adduct:

Human rbcs were treated with 2 mM phenylhydrazine for 60 min.,as described earlier. Ghosts were prepared and subjected to SDS-PAGE. One of the two parallel gels was stained and the area corresponding to the high molecular weight material at the top from the other gel, was cut and placed on top of a fresh gel. Electrophoresis was performed under the standard conditions in presence of SDS and 40 mM beta-mercaptoethanol and the gels were stained with coomassie blue. Gel A rbc membrane; Gel B high molecular weight aggregate from phenylhydrazine-treated membrane.



A B

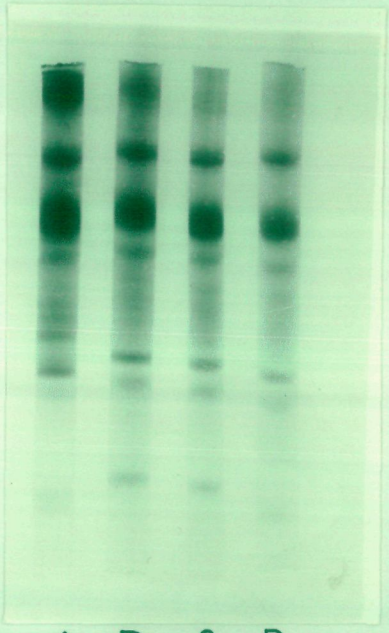
Proteolysis of the erythrocyte membrane : In order to investigate the possible contribution of proteolysis under the condition of PHZ treatment, rbc membrane were incubated at 37 deg C for various time intervals. As shown in Fig.IX, incubation at 37 deg C resulted in very significant degradation of spectrin and other polypeptides. However, no significant formation of the aggregated material could be seen on the top of the gels containing membranes incubated at various time intervals.In a preliminary study, the membranes were treated with copper-orthophenanthroline mixture which is known to cause significant oxidation of membrane sulfhydryl groups and induce cross-linking. Under such conditions there was cross-linking of spectrin and other polypeptides as well.The membranes treated with copper-orthophenanthroline mixture did not, however, show any indication of proteolysis after prolonged incubation (data not included).

PHZ administration is known to cause the production and accumulation of nydrogen peroxide (Cohen and Hochstein, 1961;1965), generation of superoxide, hydroxy and phenyl radicals (Hill and Thornalley, 1981). The hemolytic effects of PHZ have been attributed to the oxidation of sulfhydryl groups of enzymes, membrane proteins and hemoglobin (Beutler, 1969; Neely and Kraus,1972; Jain and Hochstein,1979; Jain and Subramanyan,1976; 1978).As shown in Fig.II, concentrations of PHZ which are known to decrease in vivo survival of rbcs (Jain and Hochstein, 1980),result in a very marked decrease in the membrane sulfhydryl groups. Increasing PHZ concentrations from 1 mM to 2 mM or the incubation time from 30 to 60 min. resulted in a small further decrease in the membrane sulfhydryl groups. As shown in the Fig.III, when the concentrations of PHZ, were increased upto 10 mM,over 90 % of the membrane sulfhydryl groups disappeard.Haest et al. (1981) using a number of sulfhydryl reagents observed that only about 50% of the human rbc membrane sulfhydryl groups,are non-reacting to the water soluble sulfhydryl reagents. It appears, therefore, that at high concentrations,PHZ is capable of reacting with some of these cryptic sulfhydryl groups as well. This is presumably related to partial hydrophobic nature of PHZ molecule. It is interesting to note that 2mM concentration of PHZ resulted in disappearance of about 70 % of the sulfhydryl groups, and further treatment of such membranes with iodoacetate results in loss of about 80 % of the sulfhydryl groups.This suggests that low concentrations of the PHZ treatment may modify sulfhydryl groups which are inaccessible to sulfhydryl reagents like iodoacetate, diamide, tetrathionate, etc.

The polyacrylamide gel electrophoresis of the rbc ghosts obtained

Fig. IX. Proteolysis of Human rbc Membrane.

Human rbc membranes prepared by the method of Dodge et al. (1963) as described previously, was mixed with five volumes of 5 mM-Tris acetate buffer, pH 8.0 and incubated at 37 deg C for various time intervals. The membranes were separated by centrifugation and subjected to SDS-PAGE as described earlier. Gel A Control; Gel B incubated 1 hr; Gel C incubated 2 hrs.; Gel D incubated for 3 hrs.


A B C D

IV. DISCUSSION

after treatment of rbc with 1 mM and 2 mM of PHZ, yielded patterns which are somewhat similar to those observed by Reinhart et al.(1986). As compared to the results of Reinhart et al. (1986) ghosts obtained in the present study retained much smaller amounts of hemoglobin at comparable PHZ concentrations. Hemoglobin dimers were also not observed in our studies. However,the amount of retained hemoglobin depends on the number of washes to which the ghosts were subjected to and this is responsible for the variation observed in the hemoglobin content of ghosts of various preparations.Since most of the sulfhydryl groups of hemoglobin are also modified under these conditions, presence of small amounts of hemoglobin in the ghosts may not add significantly to the membrane sulfhydryl groups. In agreement with the observations of Reinhart et al. (1986), lack of thiol reductant resulted in the smearing of bands which became sharper when beta-mercaptoethanol was incorporated in the electrophoresis buffer. This suggests some degree of formation of intermolecular disulfides. In contrast to the observation of Reinhart et al. (1986), we could locate significant coomassie blue staining material at the top of the gels, run in absence of beta-mercaptoethanol (Fig.VI and VII).This material was markedly decreased when the gels were run in presence of beta-mercaptoethanol. Loss of polypeptides corresponding to spectrin in response to PHZ treatment were observed by Jain and Hochstein (1980),in rat reticulocytes, who could also locate a high molecular weight band presumably representing the oligomeric form of spectrin. Reinhart et al. (1986) also observed, decrease in spectrin polypeptides in response to 100 mg/dl PHZ treatment in vitro,although they could not detect the formation of any high molecu-

lar weight substance. Our studies in Fig. VIII, show that the polymeric material retained on the top in response to PHZ consists mainly of spectrin and band 4.5 region. Spectrin bands readily undergo oligomerization via S-S bonds in presence of sulfhydryl cross-linking reagents (Haest et al., 1978). It is difficult to characterize the band appearing in the 4.5 region, which may be proteolyzed band 3 or polypeptide of 4.5 region itself. Indeed proteolysis is a serious problem with ghosts exposed to higher temperatures and as shown in Fig. IX, there was very significant degradation of spectrin and several other polypeptides in ghosts incubated at 37 deg C. A number of proteases have been studied in the rbc including the Ca++ activated protease (Anderson et al., 1977). However, since Ca++ is not included in the medium such a protease may not be activated to a large extent. We have also observed, in a preliminary study, that treatment with another thiol cross-linking reagent, copper-ortho-phenanthroline mixture, almost completely prevented the degradation of membrane proteins. It has been reported earlier that leupeptin, an inhibitor of thiol dependent proteinase, also inhibits calpain the major protease present in human rbc membrane (Pontremoli et al., 1984).Specific cleavage of minor components cannot be, however, completely ruled out.

V. SUMMARY

The effect of PHZ on the human erythrocyte membrane sulfhydryl groups was investigated.Treatment of erythrocytes with 1mM and 2 mM PHZ for 30 min. resulted in the loss of 60 % and 65 % membrane sulfhydryl groups respectively, as measured by the Ellman's procedure. These concentrations of PHZ cause Heinz bodies formation in human erythrocytes. Increasing of the incubation time from 30 to 60 min. resulted in small further loss of the membrane sulfhydryl groups. A concentration dependent decrease of the membrane sulfhydryl groups was observed upto 10 mM PHZ although the decrease were quite small after 2 mM PHZ.

Polyacrylamide gel electrophoresis of human erythrocyte membrane proteins treated with 1 mM or 2 mM PHZ indicated the formation of small amounts of high molecular weight aggregates that do not enter the gels. The polymeric material disappeared when the electrophoresis was performed in the presence of beta-mercaptoethanol. Re-electrophoresis of the aggregate in presence of beta-mercaptoethanol indicated the presence of spectrin polypeptides as well as a polypeptide migrating in 4.5 region. It could not be ascertained whether the latter polypeptide is the product of proteolysis of some of the larger polypeptides. Very significant degradation of rbc membrane polypeptides, especially that of spectrin,resulted when the membranes were incubated at 37 deg C. Treatment with another sulfhydryl crosslinking reagent, copper-orthophenanthroline mixture restricted such proteolysis to a very large extent.

FUTURE PLAN

It is proposed to investigate the following:

1. The nature of the sulfhydryl group of the human erythrocyte membrane proteins, that are susceptible/resistant to PHZ treatment will be investigated. Membrane will be isolated from the PHZ-treated rbc and the proteins will be fractionated either by gel filtration in presence of denaturants or by SDS-PAGE.The individual proteins will be analyzed for their sulfhydryl content. By comparison with the respective untreated samples, the extent of sulfhydryl groups modified will be determined. With the help of various sulfhydryl reagents,the nature/reactivities of the PHZ resistant sulfhydryl groups will be studied.
2. Heinz bodies will be induced in vivo by injecting appropriate amounts of PHZ in rats/rabbits. Alterations in the membrane proteins and sulfhydryl groups will be investigated and compared with those of rbcs treated with PHZ in vitro.
3. Effect of in vivo and in vitro treatment with PHZ on the rbc membrane proteolytic activity and Ca++ activated transglutaminase will be investigated.
4. The nature and extent of lipid peroxidation induced by PHZ in vivo and in vitro will be investigated and compared.

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